Gastroenterology 2019;156:1731–1741

Biomarkers Associated With Response to Regorafenib in Patients With Hepatocellular Carcinoma

Michael Teufel,[1,*] **Henrik Seidel**,[2,*] **Karl Köchert**,[2,*] Gerold Meinhardt,[1] Richard S. Finn,[3] Josep M. Llovet,[4,5,6] and Jordi Bruix[4]

[1]*Bayer HealthCare Pharmaceuticals, Whippany, New Jersey;* [2]*Bayer AG, Berlin, Germany;* [3]*David Geffen School of Medicine at UCLA, Los Angeles, California;* [4]*BCLC Group, Liver Unit, Hospital Clinic, University of Barcelona, IDIBAPS, CIBEREHD, Barcelona, Spain;* [5]*Liver Cancer Program, Division of Liver Diseases, Icahn School of Medicine at Mount Sinai, New York, New York; and* [6]*Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain*

BACKGROUND & AIMS: In a phase 3 trial (RESORCE), regorafenib increased overall survival compared with placebo in patients with hepatocellular carcinoma (HCC) previously treated with sorafenib. In an exploratory study, we analyzed plasma and tumor samples from study participants to identify genetic, microRNA (miRNA), and protein biomarkers associated with response to regorafenib. **METHODS:** We obtained archived tumor tissues and baseline plasma samples from patients with HCC given regorafenib in the RESORCE trial. Baseline plasma samples from 499 patients were analyzed for expression of 294 proteins (DiscoveryMAP) and plasma samples from 349 patients were analyzed for levels of 750 miRNAs (miRCURY miRNA PCR). Tumor tissues from 7 responders and 10 patients who did not respond (progressors) were analyzed by next-generation sequencing (FoundationOne). Forty-six tumor tissues were analyzed for expression patterns of 770 genes involved in oncogenic and inflammatory pathways (PanCancer Immune Profiling). Associations between plasma levels of proteins and miRNAs and response to treatment (overall survival and time to progression) were evaluated using a Cox proportional hazards model. **RESULTS:** Decreased baseline plasma concentrations of 5 of 266 evaluable proteins (angiopoietin 1, cystatin B, the latency-associated peptide of transforming growth factor beta 1, oxidized low-density lipoprotein receptor 1, and C-C motif chemokine ligand 3; adjusted $P \leq .05$) were significantly associated with increased overall survival time after regorafenib treatment. Levels of these 5 proteins, which have roles in inflammation and/or HCC pathogenesis, were not associated with survival independently of treatment. Only 20 of 499 patients had high levels and a reduced survival time. Plasma levels of α-fetoprotein and c-MET were associated with poor outcome (overall survival) independently of regorafenib treatment only. We identified 9 plasma miRNAs (MIR30A, MIR122, MIR125B, MIR200A, MIR374B, MIR15B, MIR107, MIR320, and MIR645) whose levels significantly associated with overall survival time with regorafenib (adjusted $P \leq .05$). Functional analyses of these miRNAs indicated that their expression level associated with increased overall survival of patients with tumors of the Hoshida S3 subtype. Next-generation sequencing analyses of tumor tissues revealed 49 variants in 27 oncogenes or tumor suppressor genes. Mutations in *CTNNB1* were detected in 3 of 10 progressors and *VEGFA* amplification in 1 of 7 responders. **CONCLUSION:** We identified expression patterns of plasma proteins and miRNAs that associated with increased overall survival times of patients with HCC following treatment with regorafenib in the RESORCE trial. Levels of these circulating biomarkers and genetic features of tumors might be used to identify patients with HCC most likely to respond to regorafenib. ClinicalTrials.gov number NCT01774344. NCBI GEO accession numbers: mRNA data (NanoString): GSE119220; miRNA data (Exiqon): GSE119221

Keywords: NGS; Predictive; Prognostic Factor; Time to Progression.

Hepatocellular carcinoma (HCC) is a heterogeneous disease that lacks molecular predictors of response to available treatments. An analysis of 10 selected biomarkers for potential response to sorafenib, a multikinase inhibitor (MKI) approved for the first-line treatment of unresectable HCC, failed to identify a treatment effect in the phase 3 SHARP study.[1] More recently, the MKI regorafenib was approved for the treatment of HCC previously treated with sorafenib, based on the results of the phase 3 RESORCE trial in which regorafenib improved overall survival (OS) and time to progression (TTP) vs placebo.[2] Predictive markers of response to regorafenib, including all common clinical markers, have not been identified in analyses of other approved regorafenib indications.[3,4]

As an MKI, regorafenib blocks the activity of multiple protein kinases involved in angiogenesis, proliferation, the tumor microenvironment, and metastasis, including vascular endothelial growth factor receptors (VEGFRs) 1–3, TIE2, KIT, RET, RAF-1, BRAF, platelet-derived growth factor receptor, and fibroblast growth factor receptor, as

***Authors share co-first authorship.**

Abbreviations used in this paper: **AFP, α-fetoprotein; ANG-1, angiopoietin 1; CI, confidence interval; HCC, hepatocellular carcinoma; HR, hazard ratio; LAP TGF-β1, latency-associated peptide of transforming growth factor beta 1; LOX-1, oxidized low-density lipoprotein receptor 1; MIP-1α, C-C motif chemokine ligand 3; miRNA, micro RNA; MKI, multikinase inhibitor; mRECIST, modified Response Evaluation Criteria in Solid Tumors; mRNA, messenger RNA; NGS, next-generation sequencing; OS, overall survival; RECIST, Response Evaluation Criteria in Solid Tumors; TTP, time to progression; VEGFR, vascular endothelial growth factor receptor.**

Most current article

0016-5085
https://doi.org/10.1053/j.gastro.2019.01.261

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Regorafenib is approved for the treatment of hepatocellular carcinoma (HCC) following sorafenib. The search for biomarkers associated with response to regorafenib has so far been unsuccessful.

NEW FINDINGS

The authors identified baseline plasma concentrations of proteins and miRNAs that were associated with overall survival time after treatment with regorafenib.

LIMITATIONS

This was an exploratory, retrospective analysis that can only be considered hypothesis generating.

IMPACT

The authors identified plasma biomarkers that might be measured to identify patients with HCC most likely to respond to regorafenib. These findings require validation.

well as tumor immunity.[5,6] Protein expression analyses in an HCC preclinical model have confirmed a complex expression pattern with regorafenib that is different from that of sorafenib.[7] This complex pattern, along with disease heterogeneity, may make the identification of biomarkers challenging.

We performed a preplanned, retrospective biomarker analysis on patients in the RESORCE trial to identify potentially predictive biomarkers of benefit to regorafenib in HCC. In the absence of established or predefined biomarkers for regorafenib, we performed a broad exploratory biomarker analysis at the DNA, RNA, and protein levels that represents a much more comprehensive approach than previous studies of regorafenib or sorafenib.

Methods

Study Design and Participants

RESORCE (NCT01774344) was a randomized, double-blind, placebo-controlled, phase 3 trial. Details of RESORCE have been published elsewhere.[2] Patients with HCC who had radiologic progression on sorafenib and tolerated sorafenib were randomized 2:1 to oral regorafenib 160 mg once daily or matching placebo plus best supportive care during weeks 1 to 3 of each 4-week cycle. Treatment continued until disease progression, clinical progression, death, or unacceptable toxicity. The primary endpoint was OS (time from randomization to death due to any cause). TTP (time from randomization to radiologic or clinical disease progression) was a secondary endpoint. Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 and modified RECIST (mRECIST) for HCC were used to measure disease progression. Adverse events were graded using the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03. All patients provided written informed consent and the trial was approved by the ethics committee or institutional review board at each center and complied with Good Clinical Practice guidelines, the Declaration of Helsinki, and applicable local laws and regulations.

Procedures: Archival Tumor Tissue

The collection of archival tumor tissue was optional in RESORCE, resulting in 68 tissue samples available for the current study as formalin-fixed paraffin-embedded blocks or as cut 5-μm sections mounted on glass slides. Of these samples, 23 were selected at random from the regorafenib group for next-generation sequencing (NGS) and 62 had sufficient RNA for tumor inflammation gene expression profiling.

Mutational analysis by NGS was carried out using the FoundationOne gene panel of 315 cancer-related genes (Foundation Medicine, Inc.) as previously described.[8] Samples for NGS were selected based on their response to regorafenib treatment (as determined by investigator assessment using mRECIST) with all available samples from responders included (12 patients with complete or partial response) and a similar number of progressors chosen at random (11 patients with progressive disease).

RNA tumor inflammation gene expression profiling was conducted using the nCounter PanCancer Immune Profiling panel v1.1 that included 770 genes covering both the adaptive and innate immune response (NanoString Technologies, Inc., Seattle, WA), as well as 6 customized genes (a 5-gene signature described as prognostic for HCC [*HN1*, *RAN*, *RAMP3*, *KRT19*, *TAF9*],[9] and *c-MET*), and 14 controls (6 positive, 8 negative). Tumor inflammation was assessed by immune cell scores derived from gene expression data using nSolver v3.0 analysis software (NanoString Technologies, Inc.).

Procedures: Plasma Samples

For plasma-based analysis, K2-EDTA plasma samples were collected at baseline from all patients enrolled in RESORCE, of which 499 of 573 samples were of sufficient quality for analysis of circulating proteins and 349 of 573 had sufficient RNA for the testing of circulating microRNA (miRNA).

Concentrations of 294 circulating proteins from 499 plasma samples were quantified using a proprietary multiplex immunoassay, DiscoveryMAP v3.3 (Myriad RBM, Austin, TX), using the Luminex xMAP system. The lower limit of quantification was defined as the concentration at which the analyte measurements demonstrated a coefficient of variation of 30%. Protein concentrations were determined from the median of at least 20 individual measures. Assays were rejected if $\geq$50% of the quality controls for a single analyte were beyond 2 standard deviations.

Expression levels of 750 circulating miRNAs and 10 controls were quantified by quantitative polymerase chain reaction (miRCURY LNA microRNA PCR kit; Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol. miRNA expression had to be measurable on a continuous scale or dichotomized by preprocessing (present vs absent) and be present in $\geq$5% of patients.

Statistical Analysis

The retrospective biomarker analyses reported here were exploratory and hypothesis generating and were not powered for statistical significance. Analyses on archival tumor samples (NGS and immune profiling) were descriptive in nature due to the small sample size. All analyses were performed with SAS version 9.2 (SAS Institute, Cary, NC) or later and R software version 3.1.0 or later.

The predictive and prognostic effects (hazard ratio [HR] and 95% confidence interval [CI]) of plasma protein and miRNA levels were evaluated using a Cox proportional hazards model using Efron and Breslow tie handling, respectively. The predictive effect was modeled as a protein–treatment interaction effect and subjected to Akaike information criterion-based selection to assess its association with OS and TTP. Models were adjusted for age, sex, Eastern Cooperative Oncology Group performance status, geographic region, α-fetoprotein (AFP) level (<400 ng/mL vs ≥400 ng/mL), extrahepatic disease, and macrovascular invasion. *P* values for HRs were corrected for multiple testing using the Benjamini–Hochberg method.

Protein levels were measured as a continuous variable: HR >1 indicates enhanced treatment benefit with regorafenib vs placebo with decreased protein levels (or reduced benefit with increased protein levels); HR <1 indicates enhanced treatment benefit with increased protein levels. Subgroup analyses were performed on proteins identified as predictive for OS and TTP (adjusted $P \leq .05$): protein concentrations were dichotomized, analyzed as quartile variables, and by subpopulation treatment effect pattern plot analyses. A patient-wise protein composite score was used to investigate whether groups of patient outliers, defined by exceptionally high or low protein levels (ie, composite scores that were substantially high [>(quartile 3 + 1.5*interquartile range)] or low [<(quartile 1 – 1.5*interquartile range)]), have a predictive potential in HCC. The protein concentration composite score, and its interaction with treatment, were used in clinical Cox models. Hierarchical cluster analysis was carried out on proteins predictive for OS.

miRNA levels were measured as continuous or dichotomized variables, with values dichotomized for miRNAs detected in ≥50% of samples. An HR >1 indicates enhanced treatment benefit with regorafenib vs placebo with increased miRNA levels, whereas an HR <1 indicates an enhanced treatment benefit with decreased miRNA levels. For dichotomized miRNAs, HR relates to the presence vs absence of miRNA (ie, an increase from no expression to detectable expression), with an HR >1 indicating enhanced treatment benefit in the absence of the miRNA. miRNAs identified as potentially predictive for OS (with a Benjamini–Hochberg-adjusted $P \leq .05$) underwent a quartile analysis. miRNA function was assessed by a correlation analysis between miRNA and messenger RNA (mRNA) expression data from The Cancer Genome Atlas and the subsequent Gene Set Enrichment Analysis.[10] The Spearman's correlation between the expression pattern of each potentially predictive miRNA and all mRNAs was calculated based on The Cancer Genome Atlas expression data from HCC tissue samples (n = 419). mRNAs were sorted by correlation, and the resulting ranked gene lists underwent a Gene Set Enrichment Analysis[11] based on curated gene sets from the Molecular Signatures Database (C2 collection; Broad Institute, Inc., Cambridge, MA).[12]

Results

Patients

Between May 14, 2013, and December 31, 2015, 573 patients were enrolled and randomized (379 regorafenib, 194 placebo) in the RESORCE trial, of whom 567 initiated treatment (374 regorafenib, 193 placebo).[2] Samples for biomarker analysis (archival tumor tissue and baseline plasma samples) of appropriate quality were available for a subset of enrolled patients (Figure 1).

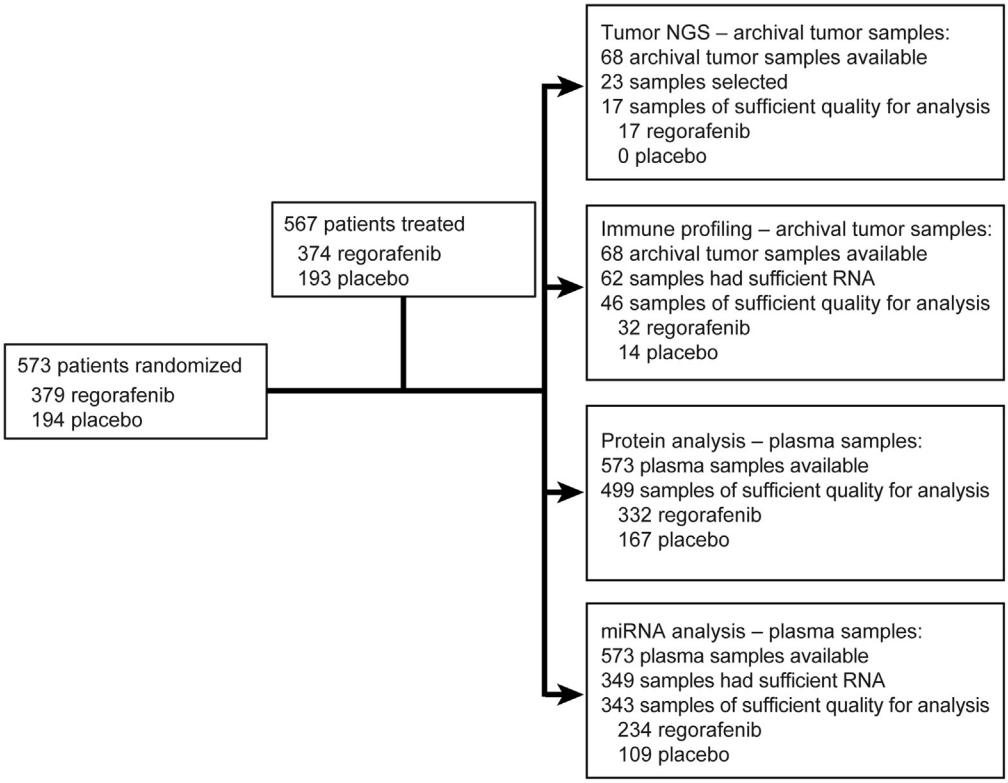

Figure 1. RESORCE patient subgroups for biomarker analyses.

Baseline demographics and disease characteristics were generally similar between the overall RESORCE patient population and the protein and miRNA cohorts, except that the miRNA cohort had a smaller proportion of Asian patients than the overall cohort (Table 1). A number of differences were observed between the overall RESORCE cohort and the NGS and immune profiling cohorts due to the small sample size in those cohorts.

Treatment outcomes (OS and TTP) in the protein and miRNA cohorts were comparable to the overall RESORCE cohort (Table 2). No statistical correlation with treatment benefit was performed for the NGS and immune profiling cohorts due to the small sample sizes.

Tumor NGS

In an effort to identify predictive genetic markers for tumor growth inhibition in response to regorafenib, mutation status in selected tumors from 12 regorafenib responders (1 complete response and 11 partial response) was compared with 11 regorafenib progressors as assessed by mRECIST. Of those, 17 had sufficient tumor content for NGS analysis (7 partial responders and 10 progressors), which revealed 49 somatic aberrations in 27 oncogenes and tumor suppressor genes, the most frequent of which were mutations in the promoter region of *TERT* (n = 9, 53% [9 of 17]), followed by mutations in *TP53* (n = 7, 41% [7 of 17]) (Supplementary Figure 1). Although gene mutations in the PI3K pathway (such as in *PTEN*, *PIK3CA*, or *TSC1/2*) were equally distributed between responders and progressors, mutations in *CTNNB1* (encoding β-catenin in the Wnt pathway) were found in 3 of 10 regorafenib progressors but not in any regorafenib responders. One of the 7 responding (and none of the progressors) patients had a *VEGFA* amplification. The 5-gene signature identified by Nault et al.[9] was identified in only 5 of the 42 samples.

Immune Profiling

Due to the potential immunomodulatory role of regorafenib, we expanded the tissue-based analysis to investigate the presence of immune gene expression signatures using a cancer immune panel from which an immune cell score was derived. Of 62 archival tumor samples, 46 passed quality control (32 regorafenib, 14 placebo). Hierarchical clustering of samples revealed 3 groups with low (21 of 46, 46%), medium (17 of 46, 37%), and high (8 of 46, 17%) immune cell scores (Figure 2*A*). In the regorafenib arm (n = 32), low, medium, and high immune cell scores were identified in 13 (41%), 13 (41%), and 6 (19%) patients, respectively (Figure 2*B*). Kaplan–Meier analysis did not reveal a difference between low and medium/high immune cell groups for median OS or median TTP; however, separation of the TTP curves at a later time point may indicate a signal worth investigating in future trials (Supplementary Figure 2). No statistical testing was done due to the small sample size.

Protein Biomarkers

In the absence of identifiable predictive genetic biomarkers, and due to the limited number of available tissue samples, expression of 294 proteins was analyzed in baseline plasma samples from 499 patients. The baseline concentrations of 112 of 266 proteins valid for analysis (28 proteins were consistently below the lower limit of detection and therefore omitted) were identified as potentially prognostic for OS (adjusted $P \leq .05$; Supplementary Table 1) and 46 of 266 for TTP (adjusted $P \leq .05$; Supplementary Table 2). The proteins prognostic for TTP largely overlapped with those prognostic for OS (35 of 46). Increased levels of both AFP (100% increase associated with HR 1.09, 95% CI 1.07–1.12; $P < .0001$) and c-MET (100% increase associated with HR 1.32, 95% CI 1.06–1.63; adjusted $P = .025$) were associated with a worse prognosis for OS. Increased AFP levels were also associated with poor prognosis for TTP (HR 1.05, 95% CI 1.02–1.07; $P = .001$). Five of 266 proteins were identified as predictive of regorafenib treatment benefit for OS (angiopoietin 1 [ANG-1], cystatin B, the latency-associated peptide of transforming growth factor beta 1 [LAP TGF-β1], oxidized low-density lipoprotein receptor 1 [LOX-1], C-C motif chemokine ligand 3 [MIP-1α]; adjusted $P \leq .05$; Table 3), with decreased levels associated with enhanced treatment benefit with regorafenib. This predictive effect was not considered to be influenced by some of the key clinical stratification and etiology variables (eg, hepatitis B and C, Child–Pugh score, alcohol use, or pattern of progression). None of the potentially predictive proteins were found to be prognostic for OS. Baseline concentrations of 47 of 266 proteins were predictive for TTP (adjusted $P \leq .05$), with all but 2 (calbindin and gelsolin) showing the same effect direction as for OS (Supplementary Table 3). The proteins predictive for TTP included the 5 proteins identified as predictive for OS. Neither AFP or c-MET were identified as predictive for OS or TTP treatment benefit. Regorafenib treatment benefit for both OS and TTP was independent of AFP and c-MET protein expression (Supplementary Table 4; Supplementary Figure 3).

A subgroup analysis was performed on the 5 proteins identified as predictive for regorafenib treatment benefit for OS to further characterize the protein concentrations at which the OS benefit may be reduced or improved. Protein concentrations were dichotomized, analyzed as quartile variables, and by subpopulation treatment effect pattern plot analyses. Overall, the regorafenib treatment benefit for OS was maintained using the different approaches, with higher protein concentrations correlating with reduced treatment benefit with regorafenib and vice versa (Figure 3; Supplementary Figure 4; Supplementary Figure 5).

Considering these results, we investigated whether there was a group of patients who possibly did not benefit from regorafenib treatment. Using a composite score approach integrating expression levels across predictive proteins (5 for OS; 47 for TTP), a small group of patients was identified (n = 20 for OS; n = 8 for TTP) with particularly high protein expression levels for whom treatment benefit with regorafenib was diminished compared with the rest of the patient groups (OS: HR 1.21, 95% CI 1.13, 1.29; $P < .0001$; TTP: HR 1.02; 95% CI 1.01–1.03; $P < .0001$). All patients

Table 1. Baseline Characteristics and Demographics for the Overall RESORCE Patient Population and Biomarker Cohorts

	Overall RESORCE cohort (N = 573)		NGS (n = 17)	Immune profiling (n = 46)		Protein (n = 499)		miRNA (n = 343)	
	Regorafenib (n = 379)	Placebo (n = 194)	Regorafenib (n = 17)	Regorafenib (n = 32)	Placebo (n = 14)	Regorafenib (n = 332)	Placebo (n = 167)	Regorafenib (n = 234)	Placebo (n = 109)
Median age, *yr* (IQR)	64 (54–71)	62 (55–68)	68 (59–73)	69 (63–73)	64 (60–68)	64 (55–71)	62 (55–70)	64 (57–71)	64 (58–70)
Sex, n (%)									
Male	333 (88)	171 (88)	14 (82)	23 (72)	13 (93)	289 (87)	148 (89)	208 (89)	95 (87)
Female	46 (12)	23 (12)	3 (18)	9 (28)	1 (7)	43 (13)	19 (11)	26 (11)	14 (13)
Race, n (%)									
Asian	156 (41)	78 (40)	7 (41)	14 (44)	3 (21)	135 (41)	63 (38)	65 (28)	23 (21)
Black or African American	6 (2)	2 (1)	0	0	0	6 (2)	2 (1)	3 (1)	2 (2)
White	138 (36)	68 (35)	9 (53)	16 (50)	9 (64)	129 (39)	65 (39)	103 (44)	46 (42)
Other/not reported	79 (21)	46 (24)	1 (6)	2 (6)	2 (14)	62 (19)	37 (22)	63 (27)	38 (35)
Geographical region, ROW, n (%)	236 (62)	121 (62)	11 (65)	21 (66)	12 (86)	209 (63)	108 (65)	178 (76)	89 (82)
ECOG PS 1, n (%)	128 (34)	65 (34)	3 (18)	9 (28)	6 (43)	119 (36)	60 (36)	74 (32)	40 (37)
AFP $\geq$400 ng/mL, n (%)	167 (44)	89 (46)	3 (18)	8 (25)	4 (29)	145 (44)	73 (44)	99 (42)	47 (43)
Extrahepatic disease, n (%)	250 (66)	132 (68)	12 (71)	24 (75)	9 (64)	223 (67)	116 (69)	158 (68)	77 (71)
Macrovascular invasion, n (%)	117 (31)	59 (30)	5 (29)	8 (25)	3 (21)	108 (33)	48 (29)	81 (35)	33 (30)
Child–Pugh score,[a] n (%)									
A5	244 (65)	118 (61)	15 (88)	23 (72)	8 (57)	210 (63)	97 (58)	146 (62)	67 (61)
A6	129 (34)	70 (36)	2 (12)	8 (25)	6 (43)	118 (36)	65 (39)	84 (36)	37 (34)
B7	5 (1)	5 (3)	0	1 (3)	0	3 (1)	5 (3)	3 (1)	5 (5)
B8	0	1 (1)	0	0	0	0	0	0	0
Hepatitis B, n (%)	143 (38)	73 (38)	6 (35)	8 (25)	2 (14)	120 (36)	58 (35)	67 (29)	30 (28)
Hepatitis C, n (%)	78 (21)	41 (21)	2 (12)	5 (16)	3 (21)	71 (21)	37 (22)	51 (22)	25 (23)
Alcohol use, n (%)	90 (24)	55 (28)	3 (18)	4 (13)	9 (64)	72 (22)	48 (29)	65 (28)	38 (35)
New intrahepatic lesions, n (%)	168 (44)	88 (45)	8 (47)	14 (44)	8 (57)	150 (45)	80 (48)	108 (46)	53 (49)
New extrahepatic lesions, n (%)	153 (40)	80 (41)	7 (41)	16 (50)	4 (29)	136 (41)	66 (40)	97 (41)	44 (40)
Progression (intra- and/or extrahepatic), n (%)	307 (81)	156 (80)	14 (82)	27 (84)	11 (79)	267 (80)	133 (80)	192 (82)	93 (85)

Variables statistics were computed according to IVRS assignments. Percentages may not total to 100% due to rounding.
ECOG PS, Eastern Cooperative Oncology Group performance status; IQR, interquartile range; IVRS, Interactive Voice Response System; ROW, rest of the world (does not include countries in Asia).
[a]The Child–Pugh score was missing for 1 patient in the regorafenib group in the overall RESORCE cohort and the protein and miRNA biomarker cohorts.

Table 2. Treatment Effect in the Overall RESORCE Patient Population vs Protein and miRNA Biomarker Cohorts

Population	n	Overall survival HR (95% CI)	Time to progression HR (95% CI)
Overall population (stratified)[a]	573	0.62 (0.50–0.78)	0.44 (0.36–0.54)
Overall population (unstratified)[a]	573	0.67 (0.55–0.83)	0.43 (0.36–0.53)[b]
Protein biomarker cohort (unstratified)[a]	499	0.64 (0.51–0.80)	0.44 (0.35–0.54)
miRNA biomarker cohort (unstratified)[c]	343	0.63 (0.48–0.83)	0.49 (0.38–0.63)

[a]Cox proportional hazards model using Efron tie handling.
[b]Using Cox proportional hazards model with Breslow tie handling this value is 0.44 (0.36–0.54).
[c]Cox proportional hazards model using Breslow tie handling.

identified for TTP (except one) were also identified for OS. This composite score group of patients was not associated with any particular patient demographic or disease characteristic (data not shown). A cluster analysis performed on the 5 predictive proteins showed variation in expression levels across 3 distinct clusters (n = 459, n = 38, n = 2), which included the 20 patients with high protein expression levels (Supplementary Figure 6).

miRNA

To gain further insights into gene expression and regulation of oncogenic pathways in HCC, we evaluated the possible regulatory role of miRNAs. Of 750 miRNAs analyzed, 25 showed a multiplicity-adjusted ($P \leq .05$) prognostic effect for OS (Supplementary Table 5). Nine showed a multiplicity-adjusted predictive effect ($P \leq .05$) for OS (Table 4): increased plasma levels of MIR30A, MIR122, MIR125B, MIR200A, and MIR374B, decreased levels of MIR15B, MIR107, and MIR320B, and absence of MIR645 were all predictive of survival benefit with regorafenib. MIR15B, MIR320B, and MIR200A were also prognostic for OS ($P \leq .05$). Similar to the protein analysis, this predictive effect was not considered to be influenced by some of the key clinical stratification and etiology variables. No miRNA was found to be predictive for TTP (data not shown). Quartile analysis of miRNA concentrations confirmed the direction of these findings (data not shown).

A functional analysis of the predictive miRNAs identified a list of 142 gene sets that showed concordance across at least 6 of the 9 predictive miRNAs (false discovery rate of 0.01). Top gene sets showed consistent patterns related to HCC subtypes and processes including liver cancer progression; metabolism of lipids, amino acids, bile acids, and xenobiotics; glucuronidation; and doxorubicin resistance (Supplementary Figure 7). The functional analysis of these top gene sets revealed that improvement in response to regorafenib could be observed in the G4/G5/G6 vs G1/G2/G3 subtypes of the classification of Boyault et al,[13] and in the CTNNB1 and polysomy 7 subtypes of the classification of Chiang et al.[14] Both of these subtypes coincide with the S3 subtype of the Hoshida classification, which is characterized in retrospective assessments by a hepatocyte-like phenotype, well-differentiated, and smaller tumors than other subtypes (S1 or S2).[15]

Discussion

In the absence of known biomarkers that predict clinical benefit for systemic treatments in HCC for MKIs, we performed a comprehensive assessment of unselected biomarkers, including genetic mutations, immune gene expression, circulating miRNA, and proteins in the phase 3 RESORCE trial of regorafenib in unresectable HCC. Our results suggest that multiple proteins and miRNAs may be predictive for OS in patients with HCC treated with regorafenib, and these results identify potential predictive biomarkers for regorafenib in this setting for the first time.

Our analysis of the association between baseline plasma levels of 266 proteins and responses to regorafenib treatment identified 5 biomarkers (ANG-1, cystatin B, LAP TGF-β1, LOX-1, MIP-1α) as possible predictors for OS, and 47 biomarkers, including the 5 predictive for OS, as possible predictors for TTP. The treatment benefit with regorafenib was maintained for the vast majority of patients in the study, with only a small proportion of patients with exceptionally high protein levels potentially deriving reduced benefit. We were unable to link this outlier group to particular patient characteristics. It is worth noting that the predictive markers identified for OS and TTP did not include well-known prognostic markers such as c-MET and AFP,[16] both of which were confirmed as being prognostic for OS. In fact, none of the 5 predictive markers for OS were identified as having prognostic relevance. Importantly, most of the predictive candidates for OS and TTP are known to play a role in inflammation and/or HCC pathogenesis, reflecting the complex etiology of HCC: LAP TGF-β1 is a precursor to TGF-β;[17] MIP-1α induces immune cell infiltration promoting liver fibrosis;[18] LOX-1 plays a role in hypoxia-induced, macrophage-derived foam cell formation and atherosclerosis,[19] which is interesting considering the immunomodulatory role of regorafenib;[6] cystatin B has been reported to be overexpressed in HCC;[20] and ANG-1 plays a role in angiogenesis and tumor progression.[21] With the exception of ANG-1, a ligand for the TIE2 receptor targeted by regorafenib, the markers have not been identified as direct regorafenib targets, although some may have an indirect connection via its immunomodulatory function.[5,6]

We explored miRNAs in this cohort to gain insight into the potential prognostic and predictive value of baseline regulation of gene expression. miRNAs are small, noncoding RNAs that regulate gene expression and are dysregulated in

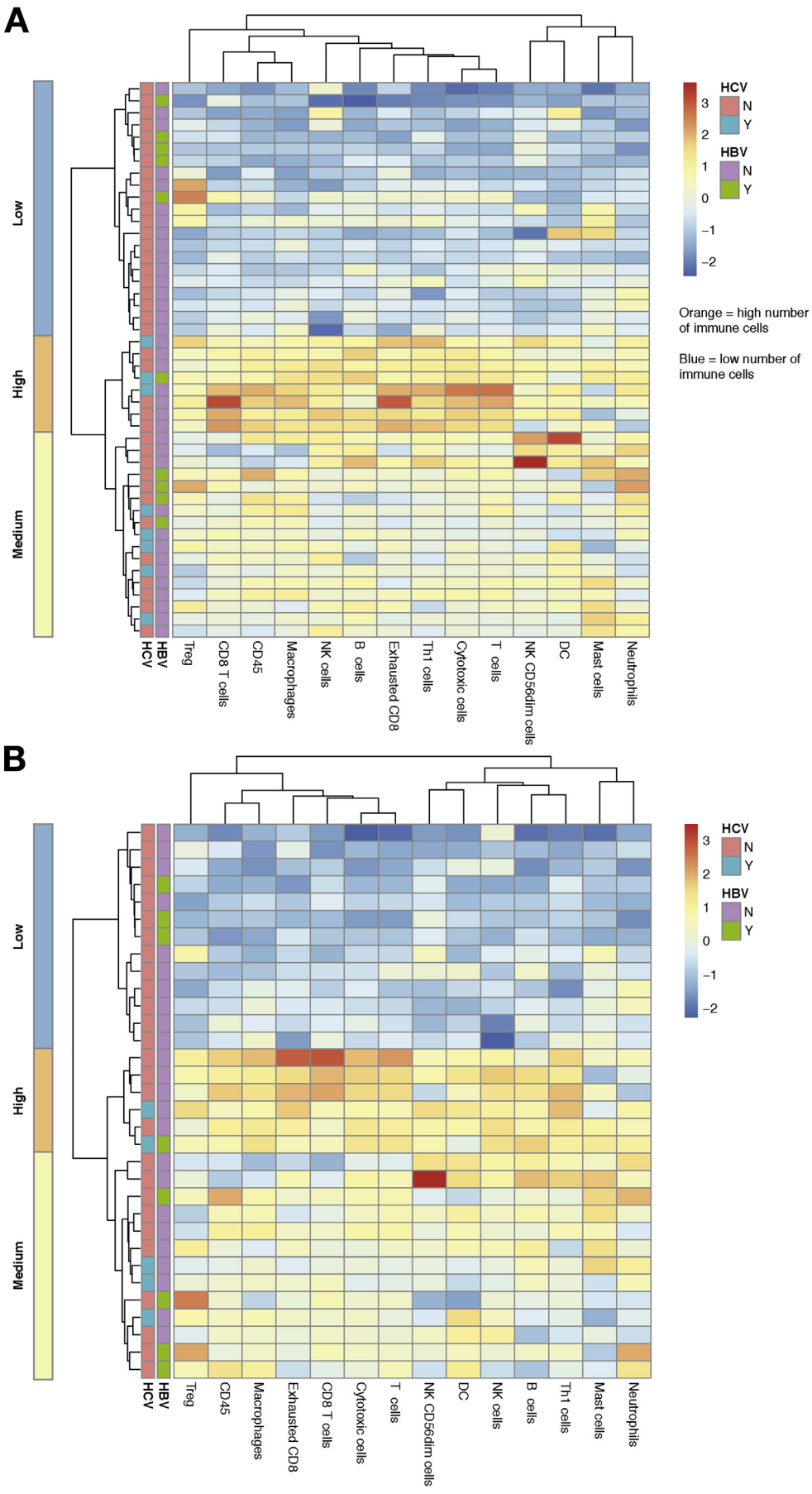

Figure 2. Hierarchical clustering of baseline gene expression data for (*A*) all 46 samples (32 regorafenib, 14 placebo) and (*B*) 32 samples from regorafenib-treated patients. DC, dendritic cell; HBV, hepatitis B virus; HCV, hepatitis C virus; N, no; NK, natural killer; Th1, type 1 T helper cell; Treg, regulatory T cell; Y, yes.

Table 3. Proteins Identified as Potentially Predictive of Regorafenib Treatment Effect on OS

	OS		TTP		
Protein	Regorafenib predictive effect, HR (95% CI)[a]	Adjusted interaction *P* value	Regorafenib predictive effect, HR (95% CI)[a]	Adjusted interaction *P* value	Reference[b]
ANG-1	1.12 (1.05–1.19)	.019	1.10 (1.04–1.17)	.017	1 ng/mL increase
Cystatin-B	1.46 (1.15–1.85)	.040	1.42 (1.14–1.77)	.018	2-fold increase
LAP TGF-β1	1.36 (1.12–1.65)	.040	1.41 (1.18–1.68)	.004	2-fold increase
LOX-1	1.35 (1.16–1.57)	.009	1.78 (1.33–2.39)	.003	1 ng/mL increase
MIP-1α	1.02 (1.01–1.04)	.040	1.02 (1.00–1.03)	.043	1 pg/mL increase

[a]An HR >1 indicates enhanced treatment benefit with regorafenib vs placebo with decreased protein levels (or reduced treatment benefit with increased protein levels).
[b]Reference shows to what unit increase the HR is related to.

many cancers, including HCC.[22] Multiple miRNAs were identified as being prognostic for HCC and predictive for OS in patients treated with regorafenib. Most of the miRNAs have not been described previously in HCC pathogenesis, with the exception of MIR122 and MIR200.[22–25] For example, MIR122 targets IGF1R, PDK4, LDHA, and GALNT10, thereby regulating RAS/RAF/ERK signaling and glycolysis, and has been reported to overcome resistance to sorafenib.[23] Interestingly, our functional analysis suggests that response to regorafenib is associated with previously described HCC molecular subtypes, with an improvement in response to regorafenib in the Hoshida S3 subgroup.[15]

It is worth noting here the results from a previously reported biomarker study derived from the STORM trial that assessed the efficacy of sorafenib in an adjuvant setting in patients with HCC following resection or ablation.[26] In this study, none of the tested biomarker candidates (pERK, VEGA, pVEGFR2, reported signatures, mutations) predicted a higher risk of recurrence or a benefit from sorafenib with regard to prevention of HCC recurrence. Nevertheless, our finding that the response to regorafenib is favorable in tumors with properties that are consistent with the S3 subtype of the Hoshida classification is interesting and may warrant further investigation.

Because HCC diagnosis algorithms do not require histopathologic confirmation of the disease, the optional collection of archival tissue resulted in limited availability of samples, precluding the identification of predictive effects for OS and TTP because a much larger sample size would be required. We therefore attempted to identify somatic aberrations associated with clinical response by mRECIST to regorafenib by comparing the mutational profile of regorafenib responders and progressors. The results of next-generation DNA sequencing were consistent with the published mutational landscape of HCC.[27,28] *TERT* and *TP53* were the most frequently mutated genes, with additional mutations identified in other genes that affect well-known pathways involved in HCC pathogenesis, including PI3K and Wnt signaling. Although 3 mutations in *CTNNB1*, encoding β-catenin in the Wnt pathway, occurred only in progressors, the small sample size precludes drawing any meaningful conclusions from these results. Although only 1 patient (a regorafenib responder as per mRECIST) had a focal *VEGFA* amplification, this finding warrants further investigation because it is the ligand for VEGFR, a target of regorafenib, and has been reported to confer particular sensitivity to sorafenib, although BIOSTORM was unable to confirm these results.[5,26,29]

Although we were unable to identify genetic biomarkers in archival tumor tissue, the immunomodulatory role of regorafenib sparked interest in evaluating the role of inflammation in HCC. Our results showed that the Pan-Cancer Immune Profiling panel can be used to identify tumors with medium/high scores for tumor-infiltrating immune cells in almost 60% of samples. However, an association between immune score and response to regorafenib was not assessed due to the small sample size. The 5-gene signature previously identified to be prognostic for survival after liver resection in patients with HCC[9] was identified in only 5 of the 42 samples, and therefore its prognostic impact was not confirmed. No conclusive results were obtained for the expression of c-MET.

The main limitation of this exploratory and retrospective study is that it can only be considered to be hypothesis generating for future trials in HCC. In addition, the small number of archival tumor samples prevented us from carrying out correlative statistical analyses. For the protein and miRNA analysis, continuous variables were used without a concentration cutoff, which limits the possible clinical interpretation of these findings; however, the improved power of continuous variables over discrete variables to detect a treatment effect was considered to be the best approach for a comprehensive, hypothesis-generating study.

Thus far, rational biomarker selection has been unsuccessful in identifying predictive markers for regorafenib in colorectal cancer and gastrointestinal stromal tumors.[3,4] The broader approach used in this study is not only biologically warranted considering the heterogeneity of HCC tumors,[15,27] but is also needed due to the multiple targets and pathways affected by MKIs such as regorafenib.[7] Recent protein expression analysis of regorafenib and sorafenib has revealed a complex and distinct pattern of protein expression, which may provide some rationale as to why regorafenib is active in sorafenib-refractory patients. Disease

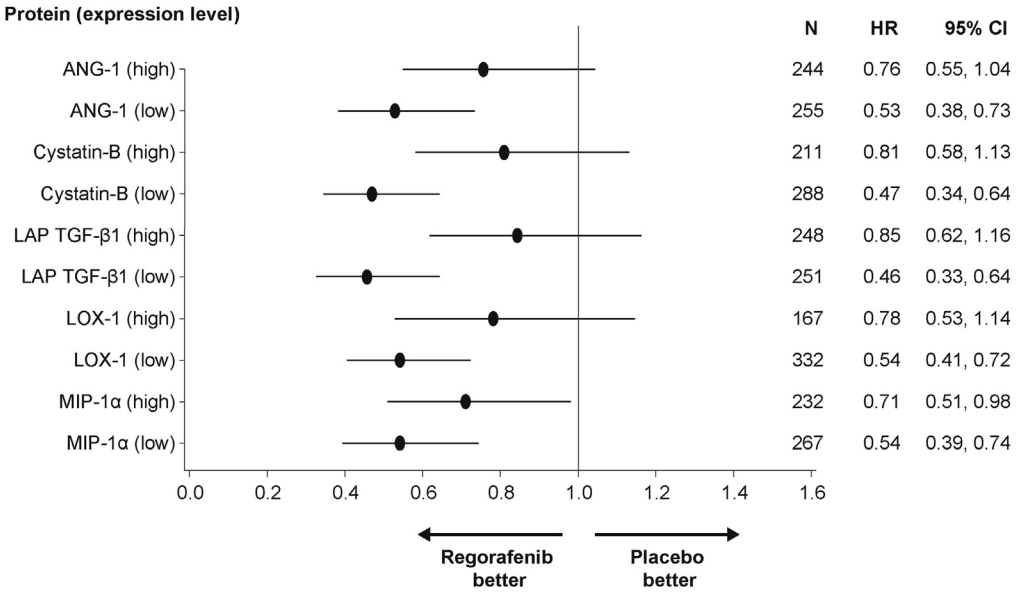

Figure 3. Protein dichotomized subgroup analysis for OS regorafenib treatment benefit. Error bars denote 95% CIs of the treatment HR. Note, the HR reference level line was set to 1. HR <1 indicates regorafenib treatment benefit. Dichotomized protein expression was categorized into high (>median) vs low (≤median). Cox models were adjusted for Eastern Cooperative Oncology Group performance status, AFP level, macrovascular invasion presence, geographical region, and extrahepatic disease presence.

heterogeneity and multikinase inhibition also make it perhaps unlikely that a single target will be identified that can predict treatment benefit. Identifying a molecular signature may be more successful and several HCC classification systems have been described, which until now have not been linked to treatment or to prognosis.[13–15]

In conclusion, this hypothesis-generating study for biomarkers predicting response to regorafenib treatment in HCC has led to the identification of protein and miRNA biomarker candidates and signatures that warrant further validation in future studies. Although AFP and c-MET are well-known prognostic markers for OS in HCC, with elevated AFP recently described to be associated with benefit to ramucirumab,[30] we found no apparent association between protein expression levels of AFP or c-MET and regorafenib treatment benefit with respect to OS and TTP, which rules them out as likely predictive biomarker candidates.

Table 4. Predictive miRNA Markers for OS and Their Prognostic Effects

	miRNA predictive for OS	
hsa-miRNA	HR (95% CI)[a]	*P*[b]
MIR15B	0.37 (0.20–0.70)	.002
MIR107	0.54 (0.37–0.81)	.003
MIR320B	0.57 (0.41–0.81)	.001
MIR122	1.35 (1.14–1.60)	.0004
MIR374B	1.36 (1.11–1.65)	.002
MIR200A	1.39 (1.15–1.68)	.001
MIR30A	1.47 (1.14–1.88)	.003
MIR125B	1.54 (1.19–1.99)	.001
MIR645[c]	3.16 (1.52–6.55)	.002

hsa, human (homo sapiens).
[a]For all miRNAs except MIR645, an HR >1 indicates enhanced treatment benefit with regorafenib vs placebo with increased miRNA levels, whereas an HR <1 indicates an enhanced treatment benefit with decreased miRNA levels.
[b]*P* values maintained significance following Benjamini–Hochberg correction for multiple testing at an adjusted $\alpha \leq 0.05$.
[c]For MIR645, which was dichotomized by preprocessing, the HR relates to the presence vs absence of the miRNA, with an HR >1 indicating regorafenib treatment benefit in the absence of miRNA.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2019.01.261.

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Received August 7, 2018. Accepted January 30, 2019.

Reprint requests
Address requests for reprints to: Michael Teufel, PhD, Bayer HealthCare Pharmaceuticals, Building B200, 2B2208, Whippany, New Jersey 07981. e-mail: michael.teufel@bayer.com.

Acknowledgments
The authors thank Georg Beckmann for his input into the study.

Editorial assistance in the preparation of this manuscript was provided by Katrin Gudmundsdottir of OPEN Health Medical Communications (London, UK), with financial support from Bayer.

Author contributions: Michael Teufel, Gerold Meinhardt, and Jordi Bruix conceived and designed the study. All authors collected, assembled,

analyzed, and interpreted the data. All authors had access to the study data and reviewed and approved the final manuscript. All authors agree to be accountable for all aspects of the work, which includes ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

The funder (Bayer) provided the study drug and worked with the principal investigator (Jordi Bruix) and the study steering committee to design the study. Data collection and interpretation, and preparation of this report, were done by the investigators and the funder. Statistical analyses were performed by the funder. All authors reviewed this report and approved the submission for publication, had full access to the data, and vouch for the completeness and accuracy of the data and adherence of the study to the protocol.

Availability of the data underlying this publication will be determined according to Bayer's commitment to the EFPIA/PhRMA "Principles for responsible clinical trial data sharing." This pertains to scope, time point, and process of data access. As such, Bayer commits to sharing upon request from qualified scientific and medical researchers patient-level clinical trial data, study-level clinical trial data, and protocols from clinical trials in patients for medicines and indications approved in the United States and European Union as necessary for conducting legitimate research. This applies to data on new medicines and indications that have been approved by the EU and US regulatory agencies on or after January 01, 2014. Interested researchers can use www.clinicalstudydatarequest.com to request access to anonymized patient-level data and supporting documents from clinical studies to conduct further research that can help advance medical science or improve patient care. Information on the Bayer criteria for listing studies and other relevant information is provided in the "Study sponsors section" of the portal. Data access will be granted to anonymized patient-level data, protocols, and clinical study reports after approval by an independent scientific review panel. Bayer is not involved in the decisions made by the independent review panel. Bayer will take all necessary measures to ensure that patient privacy is safeguarded.

Conflict of interest

Michael Teufel, Henrik Seidel, Karl Köchert, and Gerold Meinhardt are employees of Bayer. Michael Teufel, Karl Köchert, and Gerold Meinhardt own stock in Bayer. Richard S. Finn has received consultancy fees from Astra Zeneca, Bayer, Bristol-Myers Squibb, Eisai, Merck, Pfizer, Roche, Novartis, and Lilly. Josep M. Llovet reports receiving commercial research grants from Bayer Healthcare Pharmaceuticals, Eisai, Bristol-Myers Squibb, Ipsen, Blueprint, and Incyte and is a consultant/advisory board member for Bayer Healthcare Pharmaceuticals, Bristol-Myers Squibb, Eli Lilly, Eisai Inc., Celsion, Exelixis, Merck, Blueprint, Ipsen, Glycotest, Navigant, Leerink Swann LLC., Midatech Ltd., Fortress Biotech Inc., Spring Bank Pharmaceuticals, Nucleix, and Can-Fite Biopharma. Jordi Bruix has received consultancy fees from Daiichi Sankyo, ArQule, Bayer, AbbVie, Bristol-Myers Squibb, GlaxoSmithKline, Lilly, Kowa, Novartis, Roche, Onxeo, Terumo, Sanofi Aventis, and Sirtex; advisory board fees from Bayer, AbbVie, Bristol-Myers Squibb, Novartis, Roche, Onxeo, Terumo, Sirtex, MSD, and BTG; and grants from Bayer.

Funding

The study was funded by Bayer. No grant number is applicable.